

In re Application of: ITSKOVITZ-ELDOR et al
Serial No.: 10/536,734
Filed: May 27, 2005
Office Action Mailing Date: March 10, 2009

Examiner: KIM, Taeyoon
Group Art Unit: 1651
Attorney Docket: 29601

REMARKS

Reconsideration of the above-identified application in view of the amendments above and the remarks following is respectfully requested.

Claims 193-202, 205, 214-234 are in this Application. Claims 194, 201 and 216-234 have been withdrawn from consideration. Claims 193, 195-200 and 202, 205, 214 and 215 have been rejected.

35 U.S.C. § 103 Rejections

The Examiner has rejected claims 193, 195-200 and 202, 205, 214 and 215 under U.S.C. 103(a) as being unpatentable over Lumelsky et al. in view of Dang et al. in further view of Ling et al.

The Examiner has further rejected claims 193, 214 and 215 under U.S.C. 103(a) as being unpatentable over Lumelsky et al. in view of Dang et al. in further view of Thomson et al.

As was argued in the previous response, the present methodology differs from that of Lumelsky in that it includes an additional cell selection step which leads to formation of surface bound clusters. The Examiner states that although Lumelsky et al. do not teach this step, Dang et al. teach dissociation for the purpose of performing flow cytometry.

Claim 193 of the instant application actually states:

"subjecting said cells displaying at least one characteristic associated with a pancreatic islet cell progenitor phenotype to a second set of culturing conditions selected suitable for formation of surface bound cell clusters" and for "inhibiting growth of non insulin producing cells"

Although Dang et al. teach dissociation of cell, neither Dang et al. nor Lumelsky et al. teach or suggest culturing dissociated cells under such conditions, since as stated by the Examiner, Dang et al. dissociate the cells for the purpose of cell selection via flow cytometry and do not describe or suggest further culturing, nor do they provide conditions which can be used for further culturing of clusters.

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The Examiner argues that Lumelsky et al. teach a culturing condition suitable for formation of clusters and inhibiting growth of non-insulin producing cells do not have any merit, since the teachings of Dang et al. require a different set of conditions for cell dissociation. If indeed one of ordinary skill in the art were to combine the teachings of Lumelsky et al. and Dang et al. it would be for the purpose of FACS-screening dissociated cells and not for generating surface bound clusters from dissociated cells, since clearly, the conditions set forth by Dang et al. would not permit formation of such clusters (Dang et al., page 445, under "Flow Cytometry").

Although Lumelsky et al. teach a culturing condition which is suitable for generating surface bound clusters, Lumelsky et al. do not teach a step of cell dissociation, a prerequisite to cluster formation. Applying Dang et al. as teachings of ES cell dissociation does not apply, since as argued above, in dissociation, Dang et al. utilize conditions that are not suitable for cluster formation. Since Lumelsky et al. do mention the importance of cluster formation to selection or indeed link cluster formation to selection in any way, if one of ordinary skill in the art were to combine Lumelsky et al. with Dang et al. one would end up with a dissociated cell culture incapable of forming clusters.

The Examiner further states that "when there is a design need or market pressure to solve a problem and there are finite number of identified, predictable solutions, a person of ordinary skill has good reason to pursue the known options within his or her technical grasp".

Applicant agrees that indeed there are identified, predictable solutions that can be used in protocols for generating pancreatic cell precursors, however, the solution proposed by the present inventors is clearly not an identified, predictable solution.

In an article published in 2004 in the International Journal of Developmental Biology (attached herewith), Kania and Co-workers reviewed the state of the art in derivation of insulin-producing cells from ES cells. This review article identifies the four step protocol of Lumelsky et al. as being one of the first to be used for generating insulin-producing cells from ES cells.

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Kania et al. state that subsequent studies published in the wake of Lumelsky et al. could not confirm the results of the four step protocol. In an attempt to obtain positive results, researchers modified/improved the protocol of Lumelsky et al., by applying identified, predictable solutions. The five different protocols utilized by these researchers are described by Kania et al. on page 1061, left column on the bottom, and illustrated in the Figure on page 1062.


Although these published studies clearly attempted to improve the protocol of Lumelsky et al. and solve a problem, none described or suggested use of the cell dissociation, cluster-culturing step of the present invention. In fact, all these studies utilized the Nestin+ selection step while adding or modifying subsequent steps which employed various proposed pancreatic differentiation factors.

Applicant strongly believes that this review article by Kania et al. renders moot any arguments made by the Examiner as to the obviousness of the present invention. Clearly, in facing a problem to be solved, several highly skilled artisans did not modify the teachings of Lumelsky to include a dissociation and clustering step but rather used the identified, predictable solutions available to them.

As such, Applicant strongly believes that the present invention as claimed is patentable over Lumelsky and Dang et al. or Lumelsky et al. and Dang et al. in combination with others.

In view of the above amendments and remarks it is respectfully submitted that claims 193, 195-200, 202-205 and 214-215 are now in condition for allowance. A prompt notice of allowance is respectfully and earnestly solicited.

Respectfully submitted,


Martin D. Moynihan
Registration No. 40,338

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Enclosure:

- Reference: Kania et al.

The generation of insulin-producing cells from embryonic stem cells - a discussion of controversial findings

GABRIELA KANIA, PRZEMYSŁAW BLYSZCZUK and ANNA M. WOBUS*

In Vitro Differentiation Group, IPK Gatersleben, Germany

ABSTRACT The derivation of insulin-producing cells from embryonic stem (ES) cells has been controversially described. Whereas several authors showed successful differentiation of mouse ES cells into islet-like clusters, others could not confirm the results. Here, we present a detailed comparison of the various strategies used to generate pancreatic cells with respect to protocols and differentiation factors and give an explanation of the contradictory findings. It is suggested that the selection or enrichment of ES-derived nestin-positive cells should be avoided, since these cells are already committed to a neural fate before pancreatic differentiation is induced.

KEY WORDS: *mouse embryonic stem cell, differentiation, C-peptide, insulin-producing cell, nestin*

Introduction

The generation of insulin-producing cells from differentiated embryonic stem (ES) cells by a four-step protocol was described some years ago (Lumelsky *et al.*, 2001), but subsequent studies could not confirm these results. Instead, it was demonstrated that: (i) insulin immunoreactivity could occur as a consequence of insulin uptake from the medium (Rajagopal *et al.*, 2003), (ii) neuronal cells could be formed (Rajagopal *et al.*, 2003; Hansson *et al.*, 2004; Sipione *et al.*, 2004), or (iii) insulin could be released artifactually from differentiated ES cells (Rajagopal *et al.*, 2003; Hansson *et al.*, 2004). Other authors, using modified protocols with or without *Pax4* transgene expression (Blyszczuk *et al.*, 2003; Blyszczuk *et al.*, 2004) or addition of a specific PI3K inhibitor (Hori *et al.*, 2002) reported the generation of pancreatic cells characterized by glucose-responsive insulin release and some functional properties of pancreatic cells. However, until now, there has been no explanation of these contradictory findings and a critical discussion of the pancreatic differentiation protocols is lacking.

Comparison of the differentiation systems

In Protocols 1 to 4 (Fig. 1), embryoid body (EB) outgrowths were cultured in medium supplemented by insulin, transferrin, selenium and fibronectin (= ITSFn). Culture in ITSFn medium was originally developed to enrich nestin-positive cells before induction into the neuronal lineage

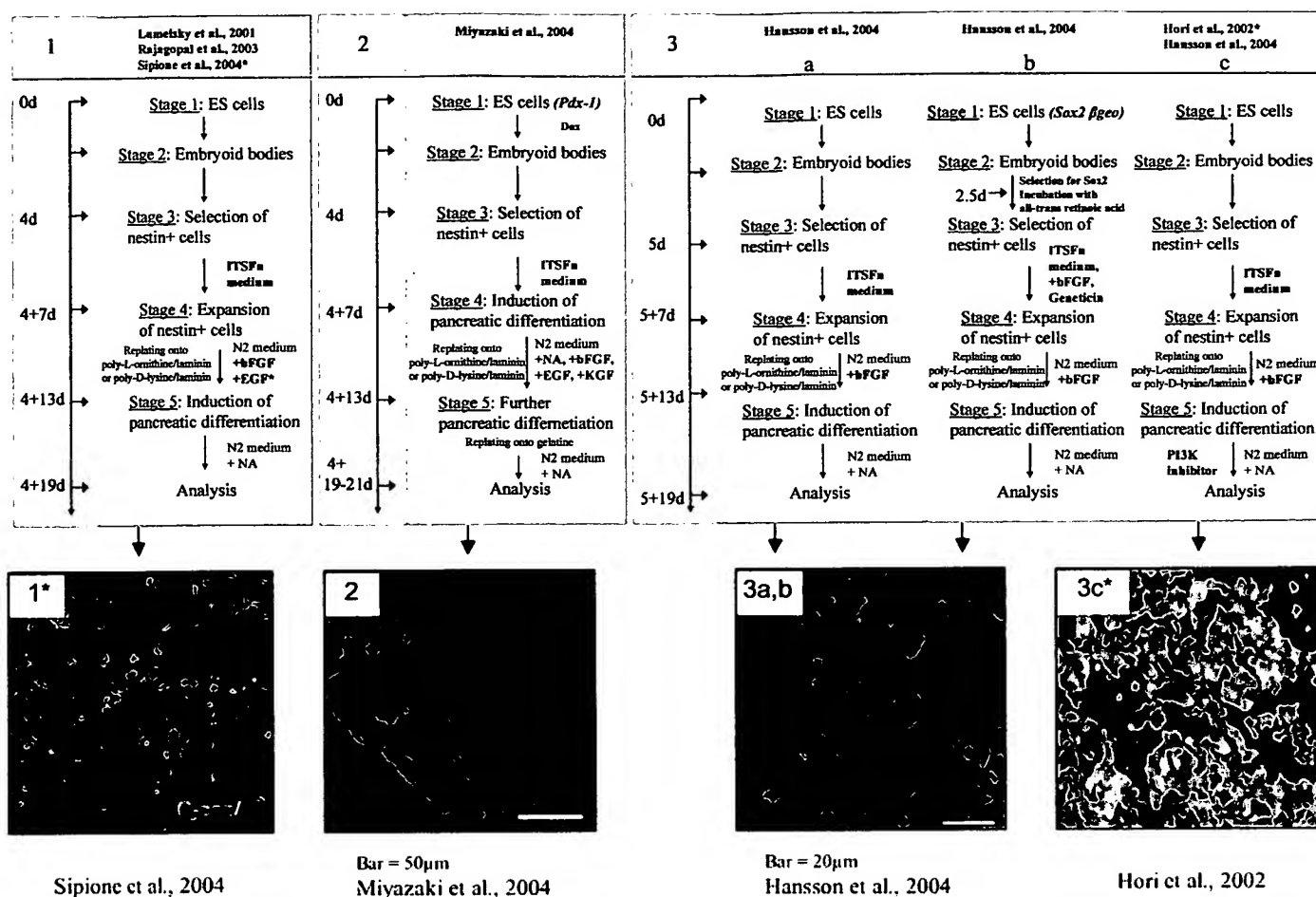
(Okabe *et al.*, 1996). In Protocols 1 to 3, proliferation of nestin-positive cells was supported by addition of bFGF (= FGF-2), but after the induction of pancreatic differentiation the FGF-2 was removed. In Protocol 2 (Miyazaki *et al.*, 2004), pancreatic differentiation had already been induced at stage 4 (with additional factors), in contrast to Protocols 1 and 3 (see Fig. 1).

In Protocols 4 (Blyszczuk *et al.*, 2003) and 5 (Blyszczuk *et al.*, 2004), the culture medium was not supplemented with FGF-2 and the differentiation time was extended to between 28 and 32 days. These cells showed insulin transcripts and C-peptide/insulin co-expression (Blyszczuk and Wobus, 2004; Blyszczuk *et al.*, 2004). ES cells constitutively expressing *Pax4* showed insulin-secretory granules (Blyszczuk *et al.*, 2003), ion channel activity of embryonal beta cells and normalization of blood glucose level after transplantation into diabetic mice (Blyszczuk *et al.*, 2004). These properties were not observed in cells which differentiated according to Protocols 1 and 3 (Fig. 1). This led us to question the role of FGF-2 in pancreatic differentiation of ES cells *in vitro*.

Pancreatic vs. neural differentiation

Following differentiation according to Protocols 1, 2 and 3, both pancreatic and neuronal cells were generated from ES cells (Lumelsky *et al.*, 2001; Hori *et al.*, 2002; Sipione *et al.*, 2004; Rajagopal *et al.*, 2003; Hansson *et al.*, 2004; Miyazaki *et al.*, 2004). As mentioned above, culture in

*Address correspondence to: Dr. Anna M. Wobus, Institute of Plant Genetics and Crop Plant Research (IPK), Corrensstr. 3, D-06466 Gatersleben, Germany.
Fax: +49-3-9482-5481. e-mail: wobusam@ipk-gatersleben.de



ITSFn- and FGF-2-supplemented medium induced commitment of cells to a neuronal fate. Also, the culture of early ES-derived cells, with a high concentration of all-trans retinoic acid (RA, 10^{-6} mol, Protocol 3b), definitely promotes neuronal differentiation of ES cells (Wobus *et al.*, 1994), whereas constitutive expression of Sox2 inhibited neuronal differentiation, but maintained properties of neural progenitors (Graham *et al.*, 2003). When cultured in the presence of FGF-2, also cells expressing the pancreatic developmental control gene *pdx-1* differentiated into the neuronal lineage (Miyazaki *et al.*, 2004).

There are several indications of a close relationship between neural and pancreatic cell types. It is well known that the transcription factors Isl-1, Ngn3, Pax6 and Pax4, neuropeptide-processing enzymes and glucose transporters are expressed in both cultured neural and endocrine pancreatic cells (Edlund, 1998; Edlund, 2001). Recently, multipotent precursor cells have been clonally isolated from adult pancreatic tissue and, when induced to differentiate, the cells developed into neural and pancreatic cell types, including neuronal and glial cells, pancreatic endocrine beta-, alpha- and delta-cells and pancreatic exocrine and stellate cells (Seaberg *et al.*, 2004). These findings suggest a close relationship of neural and pancreatic cells. We

therefore speculate that the addition of ITSFn and FGF-2 before induction of pancreatic differentiation would induce commitment of cells to a neuronal fate. However, once the cells have become committed to a neuronal fate, the procedure for induction of pancreatic differentiation eventually would activate apoptotic pathways.

FGF-2 is known to play a critical role in development *in vivo* (e.g. Joseph-Silverstein *et al.*, 1989; Kalchauer and Neufeld, 1990). *In vitro*, FGF-2 supports the proliferation and differentiation of brain-derived neural progenitor cells (Gritti *et al.*, 1995). FGF-2 is used to form neurospheres from adult neural stem cells (Reynolds and Weiss, 1992) and to generate nestin-positive cells from ES cells (Okabe *et al.*, 1996). A recent study shows that neural stem cells cultured as neurospheres in the presence of FGF-2 lose their original fate specification, resulting in modified transcript levels of regulatory genes (Hack *et al.*, 2004).

During pancreatic development, the prepancreatic endoderm is sensitive to FGF-2 concentration (Deutsch *et al.*, 2001). Whereas higher doses elicit a hepatic program, lower doses or lack of FGF-2 allow pancreatic differentiation (Kim and MacDonald, 2002). This suggests that local concentrations of FGF-2 determine the choice between a hepatic or a pancreatic fate (Rossi *et al.*, 2001) or more

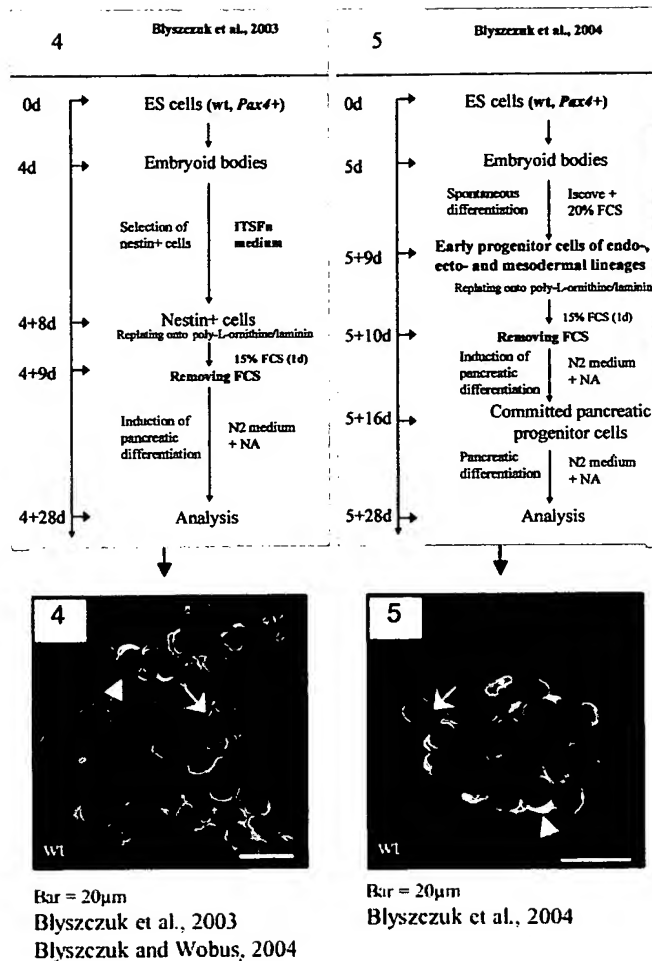


Fig. 1. Comparison of Protocols 1 to 5 used for the differentiation of ES cells into insulin-producing islet-like clusters. Images shown below show immunofluorescence labeling of insulin or C-peptide/insulin according to the following protocols: **(Protocol 1)** Based on Lumelski et al. (2001). No coexpression of C-peptide (green) with insulin (red) was found; instead, there are distinct cell populations positive either for C-peptide or for insulin (see Sipione et al., 2004). **(Protocol 2)** Based on Miyazaki et al. (2004). After pancreatic differentiation at stage 5, C-peptide-positive clusters (red) were detected, but no double labeling with insulin was obtained (Dox, doxycycline). **(Protocol 3a,b)** Based on Hansson et al. (2004). No C-peptide staining was found and insulin staining (red) was restricted to apoptotic cells. **(Protocol 3c)** Based on Hori et al. (2002). C-peptide (green)/insulin (red) co-expression (yellow) was found after the addition of the PI3K inhibitor to the differentiation medium, contrary to Hansson et al. (2004). **(Protocol 4)** Based on Blyszczuk et al. (2003). Co-expression of C-peptide (green) with insulin (red) was observed. Arrowheads indicate insulin-positive and C-peptide-negative cells (red) with small and condensed nuclei, suggesting apoptosis. These cells may represent those that take up insulin from the medium (see Blyszczuk and Wobus, 2004). **(Protocol 5)** Based on Blyszczuk et al. (2004). Coexpression of C-peptide (green) with insulin (red) was demonstrated. Arrowheads indicate C-peptide/insulin co-expression (yellow), whereas arrows indicate insulin-positive and C-peptide-negative cells (red). These cells may represent apoptotic cells, but the number of apoptotic cells in the islet-like structures was reduced compared with Protocol 4.

multi-lineage progenitor cells and following induction of pancreatic differentiation by serum-free medium containing niacinamide and laminin, functional beta-like cells were generated (Fig. 1, Protocol 5; Table 1; Blyszczuk *et al.*, 2004).

Insulin uptake and induction of apoptosis

The selection of nestin-positive cells according to Protocol 1 resulted in the activation of apoptotic pathways (Rajagopal *et al.*, 2003; Miyazaki *et al.*, 2004) and the ES-derived cells were C-peptide-negative and showed artifactual insulin release (Rajagopal *et al.*, 2003; Hansson *et al.*, 2004, see Fig. 1, Protocol 3a). C-peptide labeling of cells is an indication of proinsulin synthesis and can be demonstrated in cells that differentiate without FGF-2 according to Protocol 4 (Blyszczuk and Wobus, 2004). Only 10

generally, that different progenitor cells respond differently to FGF-2.

Protocol 5 was designed to avoid the use of ITSFn and FGF-2, so that selection or enrichment of specific cell types before induction of pancreatic differentiation would not occur. ES cells spontaneously differentiated via EBs into

TABLE 1

COMPARISON OF DIFFERENT PROTOCOLS AND PARAMETERS OF PANCREATIC DIFFERENTIATION OF MOUSE ES-DERIVED CELLS

Differentiation Protocol (see Fig. 1)	References	Insulin mRNA	C-peptide/Insulin co-expression	In vitro glucose response	In vitro C-peptide secretion	Rescue of diabetes in animal models	Electrophysiological studies	ELM studies (Insulin granules)
Protocol 1	(Lumelsky <i>et al.</i> , 2001)	+	n.d.	+	n.d.	-	n.d.	n.d.
	(Rajagopal <i>et al.</i> , 2003)	-	-	-	n.d.	n.d.	n.d.	n.d.
	(Sipione <i>et al.</i> , 2004)	+	+	+	n.d.	-	n.d.	-
			different cell populations were stained					
Protocol 2	(Miyazaki <i>et al.</i> , 2004)	+	+	-	n.d.	n.d.	n.d.	-
			(C-peptide single staining)					
Protocol 3 a,b,c	(Hansson <i>et al.</i> , 2004)	-	-	+	-	n.d.	n.d.	n.d.
Protocol 3c	(Hori <i>et al.</i> , 2002)	+	+	+	n.d.	+	n.d.	n.d.
Protocol 4	(Blyszczuk <i>et al.</i> , 2003)	+	+	+	n.d.	+	n.d.	+
Protocol 5	(Blyszczuk <i>et al.</i> , 2004)	+	+	+	n.d.	+	+	n.d.

n.d., not done

to 15% of the insulin-positive cells were not labelled by C-peptide (see Fig. 1, Protocol 4, arrow) and the cells showed small fragmented nuclei. When selective factors (such as ITSFn) were omitted in Protocol 5, the number of such potential apoptotic cells could be reduced further (see Fig. 1, Protocol 5).

We conclude that the generation of ES-derived functional insulin-producing cells without applying lineage-selection (see Soria et al., 2000; Leon-Quinto et al., 2004) is dependent on suitable differentiation protocols and differentiation factors. We therefore propose that preselection of ES-derived undefined progenitor cells by ITSFn and FGF-2 before induction of pancreatic differentiation should be omitted.

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